

## MicroReview

# Occurrence and biological function of cytochrome P450 monooxygenases in the actinomycetes

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### Summary

Many species within the order Actinomycetales contain one or more soluble cytochrome P450 monooxygenases, often substrate-inducible and responsible for a variety of xenobiotic transformations. The individual cytochromes exhibit a relatively broad substrate specificity, and some strains have the capacity to synthesize large amounts of the protein(s) to compensate for low catalytic turnover with some substrates. All three of the *Streptomyces* cytochromes sequenced to date are exclusive members of one P450 family, CYP105. In several instances, monooxygenase activity arises from induction of a P450 and associated ferredoxin, or of a P450 only, suggesting that some essential electron donor proteins (reductase and ferredoxin) are not co-ordinately regulated with the cytochrome. The overall properties of these systems suggest an adaptive strategy whose twofold purpose is to maintain a competitive advantage via the production of secondary metabolites, and, whenever possible, to utilize unusual growth substrates by introducing metabolites from these reactions into the more substrate-specific primary metabolic pathways.

### Introduction

Enzyme systems that carry out monooxygenase reactions and are based on haem proteins known as cytochromes P450 are widespread in nature but have been most actively studied in the mammalian liver because of their involvement in drug and xenobiotic metabolism. The most extensively characterized bacterial cytochrome P450 and best model system, however, is

cytochrome P450<sub>CAM</sub> from the soil bacterium *Pseudomonas putida*. P450<sub>CAM</sub> catalyses the stereo-specific hydroxylation of (1*R*)-(+)-camphor to 5-exo-hydroxycamphor, the initial step in the utilization of this substrate as a carbon and energy source (Gunsalus *et al.*, 1974; Sligar and Murray, 1986). This system differs from its mammalian counterparts in its relatively high degree of substrate specificity. In recent years the actinomycetes, particularly the genus *Streptomyces* (long known to be biologically and economically significant for the production of a diverse array of enzymes and secondary metabolites such as antibiotics) has been found to be a rich source of xenobiotic metabolizing cytochromes P450. This is hardly surprising given the number of studies documenting the ability of these organisms to perform novel and often highly selective chemical modifications (Peczynska-Czoch and Mordarski, 1984). The *Streptomyces* P450s have a relatively broad substrate specificity and may represent more accurate bacterial models for the eukaryotic cytochromes P450 than P450<sub>CAM</sub>. Also, in natural ecosystems, xenobiotic oxidation products are often more susceptible to further enzymatic breakdown either by the same or other microorganisms, leading to total biodegradation of the molecule. So the significance of the monooxygenase reactions that are the topic of this review extends from the basic biological models of xenobiotic detoxification to the practical aspects of drug and/or antibiotic production, and to reactions involved in the environmental degradation of pollutants.

### The P450 monooxygenase reaction

Most bacterial cytochromes P450 are soluble, monomeric ~45 kDa proteins containing one ferriprotoporphyrin IX (haem) prosthetic group. One of the axial ligands to the haem iron is a cysteine-sulphydryl group. When the other axial ligand of the ferrous haem iron is carbon monoxide, this co-ordination is responsible for the distinct visible absorption band at 450 nm from which these proteins take their name. In the monooxygenase reaction, two reducing equivalents reduce one atom from O<sub>2</sub> to water, while the remaining oxygen atom is incorporated into substrate, typically as a hydroxyl group (see Fig. 1). NAD(P)H

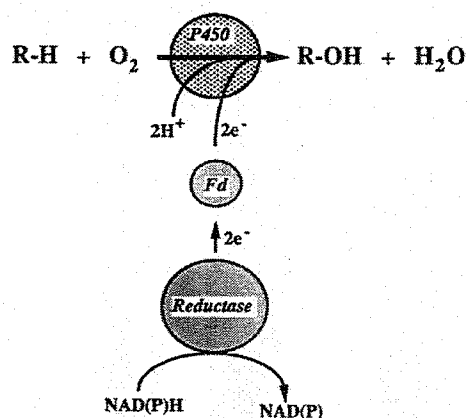


Fig. 1. Chemistry of the monooxygenase reaction and the proteins involved. R-H represents a generalized substrate which is hydroxylated to R-OH by the P450-mediated reaction. While this drawing is based on the P450<sub>CAM</sub> system, the identity and specificity of the proteins introducing the reducing equivalents to the P450 in some actinomycetes are not as clearly defined (see text).

is the usual physiological source of these reducing equivalents, which are transferred sequentially via two additional proteins, a reductase and a ferredoxin, and finally to the P450. An exception to this general description, and outside the scope of this review, is the catalytically self-sufficient P450 of *Bacillus megaterium*, containing both

reductase and cytochrome functions on one 120 kDa polypeptide (Narhi and Fulco, 1987).

A practical experimental consideration for the biochemical study of bacterial P450s is that while the cytochrome is often readily identified and purified, restoration of activity and actual characterization of the physiological role of the protein require a source of reducing equivalents. Substrate recognition and oxygen chemistry are functions that are solely the domain of the cytochrome, and substitution of alternative electron donors need not affect the chemical (substrate) specificity of the system. This substitution can be accomplished in several ways, notably by substitution of a similar reductase and Fd (Shafiee and Hutchinson, 1987), complementation of protein extracts (O'Keefe *et al.*, 1988), chemical redox mediation between a reductant and the P450 (Elbe and Dawson, 1984), and use of oxygen donor compounds such as organic peroxides to substitute for both reducing equivalents and O<sub>2</sub> (Coon and White, 1980).

#### Cytochromes P450 in the actinomycetes

Although there have been many reports of cytochrome P450-mediated reactions in the order Actinomycetales in the past several years (Table 1) the sulphonylurea herbicide-inducible cytochromes P450<sub>SU1</sub> and P450<sub>SU2</sub> from the soil bacterium *Streptomyces griseolus* provide one of

Table 1. Cytochromes P450 in the Actinomycetales.

Strain	Proteins identified	Genes identified	Inducer	Substrate	References
<i>Streptomyces griseolus</i> ATCC 11796	P450 <sub>SU1</sub> P450 <sub>SU2</sub> P450 <sub>CON</sub>	<i>suaC</i> <i>subC</i>	Sulphonylureas, phenobarbital Same as above Unknown	Sulphonylureas, phenobarbital Same as above Unknown	O'Keefe <i>et al.</i> (1988) Omer <i>et al.</i> (1990) Harder <i>et al.</i> (1991)
<i>Streptomyces griseus</i> ATCC 13273	P450 <sub>BOY</sub>		Soybean flour, genistein	7-ethoxycoumarin, precocene II	Sariaslani and Kunz (1986) Trower <i>et al.</i> (1990)
<i>Streptomyces carbophilus</i> SANK 62585	P450 <sub>SCA-1</sub> P450 <sub>SCA-2</sub>		Compactin Compactin	Compactin Compactin	Matsuoka <i>et al.</i> (1989)
<i>Streptomyces flavovirens</i> SANK 63684			Unknown	Compactin	Manome and Hoshino (1989)
<i>Streptomyces setonii</i> ATCC 39116			Veratrole, guaiacol	Veratrole, guaiacol	Sutherland (1986)
<i>Streptomyces</i> species SA-COO		<i>chaP</i>	Unknown	Unknown	Horii <i>et al.</i> (1990)
<i>Streptomyces flavovirens</i> No.28			Unknown	Phenanthrene	Sutherland <i>et al.</i> (1990)
<i>Saccharopolyspora erythraea</i> CA-340	P450 <sub>I</sub> P450 <sub>II</sub> (CYP107)	<i>eryF</i>	Unknown Unknown Unknown	7-ethoxycoumarin 6-deoxyerythronilide	Shafiee and Hutchinson (1987; 1988) Weber <i>et al.</i> (1991)
<i>Nocardia species</i> NH1	P450 <sub>NPd</sub>		<i>p</i> -alkylphenyl ethers	<i>p</i> -alkylphenyl ethers	Cartwright <i>et al.</i> (1971); Cartwright and Broadbent, (1974)
<i>Rhodococcus rhodochrous</i> ATCC 19067	P450 <sub>OCT</sub>		<i>n</i> -octane	<i>n</i> -octane	Cardini and Jurtshuk (1968; 1970)
<i>Rhodococcus</i> species			Camphor	Camphor	Chapman <i>et al.</i> (1963; 1966)

the more complete biochemical and genetic characterizations to date. These two cytochromes and their corresponding ferredoxins have been purified to homogeneity, and the genes encoding each were cloned and sequenced (O'Keefe *et al.*, 1988; 1991a; Omer *et al.*, 1990). Both cytochromes were shown to be induced by sulphonylurea (and some other) herbicides as well as phenobarbital, and they also exhibit similar yet distinct catalytic properties (Romesser and O'Keefe, 1986; O'Keefe *et al.*, 1988; Harder *et al.*, 1991). A third cytochrome, P450<sub>CON</sub>, was present independently of sulphonylurea addition to the medium, but it is not involved in herbicide metabolism and has not been characterized further (O'Keefe *et al.*, 1987; 1988).

In several of the systems described (*Streptomyces setonii*, *Nocardia* sp., *Rhodococcus rhodochrous*, and *Rhodococcus* sp., see Table 1), the metabolic step(s) carried out by cytochrome P450 successfully introduces the compound into a primary metabolic pathway, since the substrate can be utilized by the organism as a sole carbon source. *S. setonii*, for example, contains a P450 system(s) that catalyses a two-step demethylation of veratrole (a product of lignin degradation) to catechol, which is then completely metabolized via the  $\beta$ -ketoadipate pathway (Sutherland, 1986). In the majority of systems described in Table 1, P450 is specifically induced by the substrate(s) it ultimately acts upon. Although the substrate range has not been determined for all, the available evidence suggests that many of the P450s are able to metabolize a variety of substrates. Some compounds involved are generally considered as xenobiotics, which are loosely defined as compounds of natural or synthetic origin which are foreign to the organism's 'normal' chemical environment (Peczynska-Czoch and Mordarski, 1984). Since the normal environment of most of these strains is probably not static or clearly defined, we will restrict the meaning of xenobiotic to compounds synthesized and/or introduced by humans (e.g. petroleum products, pesticides). Microbial transformation of these compounds is particularly noteworthy since the enzymatic mechanisms for their metabolism have not had an opportunity to evolve into a highly efficient system for their utilization as growth substrates. In most strains involved in xenobiotic metabolism (*Streptomyces griseolus*, *Streptomyces griseus*, *Streptomyces carbophilus*, and *Streptomyces flavovirens*), the P450 catalyses a single step of substrate modification but, unlike the *S. setonii* metabolism of veratrole, the xenobiotic metabolites are not degraded further, presumably because other more substrate-specific enzymes are unable to catalyse further metabolic transformations.

The actinomycetes are well known for their ability to synthesize a wide variety of secondary metabolites, typically in conjunction with morphological differentiation,

when growth conditions are sub-optimal. Since in their natural environment they must compete with other microorganisms for limited nutrients, the ability to respond to environmental change by altering their development and by producing secondary metabolites, including antibiotics, may offer a survival advantage. One organism in which the role of a P450 in these processes has been demonstrated is *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*), in which a P450 (CYP107) encoded by the *eryF* gene has been found to catalyse the hydroxylation of 6-deoxyerythronolide B, an intermediate in the biosynthesis of a macrolide antibiotic, erythromycin A (Weber *et al.*, 1991). While it was previously thought that this hydroxylation was carried out by P450<sub>I</sub> and/or P450<sub>II</sub> (Shafiee and Hutchinson, 1987; 1988), recent results suggest that these proteins are not involved in erythromycin biosynthesis (J. Andersen and C. R. Hutchinson, personal communication). Alternatively, the finding that *S. erythraea* can utilize compounds such as octane and guaiacol as sole carbon sources suggests that a degradative role exists for these or other P450s in this strain (P. A. Harder, unpublished).

#### Electron transfer components of the P450 systems

In the archetypical P450<sub>CAM</sub> system, the flavoprotein reductase, [2Fe-2S]-ferredoxin, and P450 are co-ordinately regulated and cotranscribed (Koga *et al.*, 1989). Point mutations in any of the three genes in this system result in a strain unable to metabolize camphor, suggesting that there are no other proteins in the cell that can substitute for the electron-transfer functions of the reductase and ferredoxin (Rheinwald *et al.*, 1973). There are limited examples of characterized proteins involved in transfer of electrons to the actinomycete P450s, but, because of the prevalence in many bacteria of ferredoxin reductases and especially of ferredoxins of undefined physiological function, conclusions about whether a group of proteins constitutes a specific monooxygenase system should be carefully considered.

Two ferredoxins associated with the P450<sub>SU1</sub> and P450<sub>SU2</sub> monooxygenases in *S. griseolus* are small (64 and 68 amino acids), and contain a somewhat unusual single [3Fe-4S] cluster. The coding region for each of the *S. griseolus* ferredoxins is located immediately downstream from the stop codon of its associated P450, and these ferredoxins accumulate under the same well-defined induction conditions as the cytochromes (O'Keefe *et al.*, 1991a). These findings suggest that the P450-Fd pairs are cotranscribed, demonstrating that there is a well-defined relationship between the P450 and its cognate Fd (Fd-1 with P450<sub>SU1</sub> and Fd-2 with P450<sub>SU2</sub>). Although a third open reading frame located ~250 bp downstream of each Fd gene is cotranscribed with the

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### Induction, substrate turnover, and P450 accumulation

One notable feature of the P450s involved in xenobiotic metabolism is their substrate inducibility, which sometimes leads to accumulation of large amounts of P450 (Harder *et al.*, 1991), ensuring that even poor substrates are entirely converted to metabolite. A critical examination of the physiological significance of the P450-mediated metabolism of any individual substrate is possible if the rate of substrate turnover is compared with saturating concentrations of both a good substrate (electron transfer limited) and a marginal substrate (substrate limited). The turnover number, reported in reciprocal time units ( $s^{-1}$  or  $min^{-1}$ ), indicates how often a P450 molecule catalyses formation of a product molecule. An *in vitro* turnover number of  $\sim 1200\ min^{-1}$  for the P450<sub>CAM</sub> system metabolizing camphor probably reflects an upper limit for a system that has an optimized enzyme-substrate recognition upon which the organism depends as a sole carbon source (Gunsalus *et al.*, 1974). In contrast, the battery of xenobiotic-metabolizing P450s in mammalian liver has maximum electron transfer limited turnover numbers of  $\sim 60\ min^{-1}$ , although values much less than  $1\ min^{-1}$  for xenobiotic substrates are frequently observed (Guengerich *et al.*, 1982). Under optimal conditions with good substrates, reconstituted *S. griseolus* P450<sub>SU1</sub> has a turnover number of  $\sim 20\ min^{-1}$ , probably limited by the electron-transfer rate to the P450. With chlorimuron ethyl as substrate, the value is much lower ( $<0.01\ min^{-1}$ ). In spite of this, chlorimuron ethyl is effectively metabolized by P450<sub>SU1</sub> in whole cells (O'Keefe *et al.*, 1991a). While a low turnover number makes it virtually impossible to demonstrate that the enzymatic reaction is catalytic (multiple turnover) *in vitro*, it also makes it difficult to assess the role of associated electron-transfer proteins unless a high turnover substrate is identified. A very low turnover ( $<0.002\ min^{-1}$ ) was reported for the *S. erythraea* reductase-Fd-P450 system (Shafiee and Hutchinson, 1987; 1988), but recent experiments reveal that this was because the true 6-deoxyerythronolide B hydroxylase P450 (CYP107, Weber *et al.*, 1991), with a turnover of  $\sim 66\ min^{-1}$ , was only a very minor component of the preparation (J. Andersen and C. R. Hutchinson, personal communication). The enzymes from *S. griseus* and *S. carbophilus* metabolize some substrates at  $>1\ min^{-1}$  (Matsuoka *et al.*, 1989; Trower *et al.*, 1988; 1990a), although a large number of substrates for *S. griseus* P450<sub>soy</sub> have been reported without quantification (Trower *et al.*, 1988).

Levels of P450 accumulation in *S. griseolus* exhibit an inverse correlation with the enzyme turnover number for that inducer/substrate (Harder *et al.*, 1991). With compounds that are rapidly metabolized, levels of P450 in total soluble extract are low; with slow turnover, and espe-

cially when *in vivo* turnover is inhibited by aniline or 1-phenylimidazole, P450 levels are higher. This is probably because the inducer/substrate is available and able to elicit an induction response for a longer time. Genetic analysis of the *S. griseolus* P450 systems, including the finding that expression of P450<sub>SU1</sub> and Fd1 was constitutive in both *S. lividans* and *suaB,C* deletion mutants transformed with the *suaB,C* genes, suggests that these genes are negatively regulated and that regulation occurs at the level of transcription, presumably by reversible inducer interaction with a repressor protein (O'Keefe *et al.*, 1991a; Harder *et al.*, 1991; N. Patel and C. A. Omer, in preparation). Preliminary experiments indicate that both good and marginal substrates elicit similar levels of mRNA in *S. griseolus* (P. A. Harder, unpublished), demonstrating that the ability of many sulphonylureas to stimulate transcription is relatively equal, or at least does not vary over several orders of magnitude (as their rate of metabolism does). Examples of compounds that can act as gratuitous inducers (non-metabolized) or non-inducing substrates (Harder *et al.*, 1991) further suggest that the inducer specificity of the putative repressor molecule is not identical to that of the P450 active site, although the specificities of both are relatively broad.

### Cytochrome P450 protein sequences

The DNA sequences are available for three of the *Streptomyces* cytochrome P450 genes (Omer *et al.*, 1990; Horii *et al.*, 1990). The *suaC*, *subC*, and *choP* genes are named in the systematic P450 nomenclature CYP105A1, CYP105B1, and CYP105C1, respectively (Nebert *et al.*, 1987). The designation CYP105 places them within the same gene family since their amino acid sequences are  $>36\%$  identical to each other, but  $<36\%$  identical to any other P450s. In contrast, the *eryF* gene product from *S. erythraea* has been placed in the CYP107 family (Weber *et al.*, 1991). Sequence alignment of the three *Streptomyces* proteins shows that the identity between the two *S. griseolus* cytochromes, P450<sub>SU1</sub> and P450<sub>SU2</sub> (44.4%), is within 1% of that between P450<sub>SU1</sub> and the *choP* gene product (43.7%) and between P450<sub>SU2</sub> and the *choP* gene product (43.5%). The similarity among these proteins could manifest itself in distinct common secondary and/or tertiary structural features. The availability of the crystal structure of P450<sub>CAM</sub> (Poulos *et al.*, 1987), although  $<30\%$  identical in aligned sequence, enables mapping of these common *Streptomyces* sequence features to the three-dimensional structure of P450<sub>CAM</sub>.

It is evident from various studies that the N-terminal region of the P450 is poorly conserved (see Fig. 2; Nebert *et al.*, 1987), even among proteins from the same strain performing similar functions, like the *S. griseolus* P450<sub>SU1</sub> and P450<sub>SU2</sub>. Since this information is readily obtained

from a purified protein, its usefulness should not be underestimated as a means of obtaining a 'fingerprint' of the P450 under study. Further inspection of Fig. 2 reveals that as the C-terminus is approached the percentage of identical residues in the three sequences increases: 16% in amino acids 1–100, 31% in 101–200, 38% in 201–301, and 47% in 301–400. Distinct patches of conserved residues can be seen as well, and those with clearly defined relationships to function have been described at length (Omer *et al.*, 1990; O'Keefe *et al.*, 1991b). It was concluded that the region around Cys-357 (residues 350–360) is involved in providing a 'pocket' for the cysteine haem co-ordination, and residues 241–252 is a region of  $\alpha$ -helix near the oxygen co-ordination site on the other side of the haem. While it is not surprising that some of the strongly conserved sequences among these proteins fall in regions that map to critical structural features of P450<sub>CAM</sub>, more unexpected is the strong conservation of other helical regions not clearly associated with any essential function. The first of these (residues 145–167) is strongly conserved (75% identical) and maps exactly to an  $\alpha$ -helical region of P450<sub>CAM</sub>, and the second (262–289) maps to two other  $\alpha$ -helical regions. The residue 145 to 167 stretch may form a surface-exposed amphiphilic helix that is the most identifiable unique feature of the *Streptomyces* P450s.

### Conclusions

The total number of P450 enzymes that any individual actinomycete strain may be able to synthesize has not been determined. The largest number has been detected in *S. griseolus*, in which there is evidence at the protein level for four distinct P450s (O'Keefe *et al.*, 1988; D. P. O'Keefe, unpublished). Detection of the protein depends on both expression and accumulation, which is a complex function of growth conditions, induction, and inducer metabolism. There is no evidence that any of these P450s are involved in essential metabolic functions. Within the CYP105 family, cross-hybridization of a P450 gene with genomic DNA has been used to identify a related gene (Omer *et al.*, 1990), but the homology among other P450 families potentially present may not be sufficient to identify all P450s in the genome, and identification strategies based on more specific sequence homologies may be required.

It seems possible, given the presence in these strains of ferredoxins of ill-defined but apparently essential function (Donadio and Hutchinson, 1991), that some P450s may be synthesized alone. While *S. griseolus* responds to the presence of sulphonylurea inducers with the production of two P450s and associated ferredoxins, in the *Streptomyces* sp. *choP* system, no associated Fd is evident. Although it cannot be ruled out, there is currently no

evidence in the *Streptomyces* for co-ordinate induction of an entire system of reductase, ferredoxin, and P450, as is the case for P450<sub>CAM</sub>. It is worth considering that the *in vivo* ferredoxin reductase function may not be served by the 'traditional' flavoprotein NADPH:Fd reductase, but is a secondary role of some other enzyme which can divert reducing equivalents from a different redox enzyme system.

The actinomycetes differ from many prokaryotes in their ability to exhibit highly variable phenotypes, including undergoing complex morphological differentiation and producing a multitude of secondary metabolites, generally under conditions that are not favourable for growth. For organisms that thrive in environments of diverse nutrient input, the ability to produce a variety of broad-specificity enzymes may represent part of an overall strategy to cope with a constantly changing environment (Hopwood and Merrick, 1977). With this in mind, the roles of the P450s described here seem clearer; the P450 can participate in a multi-enzyme pathway to synthesize a 'useful' secondary metabolite (e.g. an antibiotic), or to incorporate an exogenous compound into a primary metabolic pathway. When the compound is a xenobiotic as defined earlier, neither a useful metabolite nor introduction into a primary pathway occurs. Chemical modification of the xenobiotic by these inducible enzymes is a result of its ability to interact with factors controlling transcription, and the broad substrate specificity of the P450 itself. In *S. griseolus*, some marginal P450 xenobiotic substrates are good inducers, initiating a cycle whereby the accumulation of protein is amplified by the slow metabolism of the inducer. The purpose of such a mechanism may be to adjust the level of a single broad-specificity enzyme to optimize metabolism of both good and poor substrates.

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